Perinatal asphyxia and intervention: possible neuroprotective effects of intravenous nicotine administration.

An experimental study in newborn piglets

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To my Godchildren

Maria, Ingvild & Benedicte

And to Jonas

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Publications included in the thesis

I **Andresen JH, Solberg R, Løberg EM, Munkeby BH, Stray-Pedersen B, Saugstad OD**

Resuscitation with 21 or 100% Oxygen in Hypoxic Nicotine-Pretreated Newborn Piglets: Possible Neuroprotective Effects of Nicotine. Neonatology 2008 93:36-44

II **Andresen JH, Godang K, Munkeby BH, Stray-Pedersen B, Saugstad OD**

Nicotine in a small to moderate dose does not cause a significant increase in plasma catecholamine levels in newborn piglets. Neonatology 2008 94:279-283

III **Andresen JH, Saugstad OD**

Effects of nicotine infusion on striatal glutamate and cortical Non-Protein Bound Iron in hypoxic newborn piglets. Neonatology 2008.94:284-292

IV **Andresen JH, Løberg EM, Wright M, Goverud IL, Stray-Pedersen B, Saugstad OD**

Nicotine increases the expression of Brain-Derived Neurotrophic Factor mRNA and protein in the hippocampus of hypoxic newborn piglets. Submitted.

Abbreviations

A	adrenaline, epinephrine
aEEG	amplitude-integrated Electroencephalogram
AIF	apoptosis inducing factor
BDNF	brain derived neurotrophic factor
BE	base excess
CBF	cerebral blood flow
CNS	central nervous system
CSF	cerebrospinal fluid
ERK	extracellular signal-related kinase
FiO_2	inspired fraction of oxygen
GC-MS	gas chromatography-mass spectrometry
H&E	Hematoxylin and Eosin
HIE	hypoxic ischemic encephalopathy
HPLC	high performance liquid-chromatography
HR	heart rate
kDa	kilo Dalton
LLA	lower level of cerebral autoregulation
MABP	mean arterial blood pressure
MAP-2	microtubule-associated protein 2
MW	molecular weight
nAChR	nicotinergic Acetylcholine receptors
NA	noradrenaline, norepinephrine
NPBI	non-protein bound iron
PCR	polymerase chain reaction

1. Introduction

1.1 Perinatal asphyxia

1.1.1 Definition

Asphyxia features hypercapnia (increased levels of carbon dioxide in blood), hypoxemia (low oxygen concentration in arterial blood), and ischemia (diminished amount of blood perfusing the brain) (1). There is little agreement over the clinical definition of perinatal asphyxia, but there is consensus on the fact that no single feature alone should be used as definition. Two main characteristics of perinatal asphyxia are signs of cardiorespiratory and neurological depression, seen on a low Apgar score (≤ 3 at five minutes or later) (2) and metabolic acidosis. Metabolic acidosis is often defined as an umbilical arterial cord pH of < 7.0 and/or BE < -12 mmol/l (UK) or < -16 mmol/l (US and Canada) (3-5).

1.1.2 Incidence

Due to the lack of coherence regarding definitions for perinatal asphyxia, it is difficult to make accurate estimates of the incidence. It also varies between developed countries and resource poor countries. In our part of the world the incidence for severe perinatal asphyxia (causing death or severe neurological impairment) is approx. 1/1000 live births, as opposed to 5-10/1000 live births in developing countries (6). According to the 2004 World Health Organization report, perinatal asphyxia causes 23% of the 4 million neonatal deaths worldwide (7).

1.1.3 Aetiology

Perinatal asphyxia may occur antenatal (20%), intrapartum (35%), intra- and antepartum (35%), and immediately postnatal (10%) (1). Causes are numerous, all

leading to impaired cerebral blood flow. Mostly this impaired cerebral blood flow occurs as a consequence of interruption of placental blood flow and gas exchange (e.g. umbilical cord compression, anaemia, bleeding, congenital cardiac and pulmonary anomalies, uterine hyperactivity, placental abruption, and birth trauma). The main risk factors for postnatal asphyxia are maternal opiates causing respiratory depression, obstructed airways or congenital sepsis (5, 6).

1.1.4 Diagnosis and prognosis

Perinatal asphyxia is diagnosed by a combination of symptoms and biochemical findings. Apgar score has been used to assess the newborn's clinical condition since it was published by Virginia Apgar in 1953 (8). It is however a poor predictor of outcome, and should always be accompanied by other criteria (2). Other non-specific signs of asphyxia include metabolic acidosis and meconium staining of the amniotic fluid.

To diagnose perinatal asphyxia one should use the combination of low Apgar score, biochemical indicators (pH and BE), and clinical signs of hypoxic-ischemic encephalopathy (HIE) and multiple organ affection. HIE is graded according to a system introduced by Sarnat and Sarnat in 1976 (9), and modified by Levene et al in the 1980's (10). It consists of three clinical stages, and is characterised by a pattern of evolving neurological signs over the first few days of life. The first clinical stage, mild HIE, is characterized by hyperalertness, staring, normal or decreased spontaneous motor activity, and a lower threshold for all stimuli. Stage two, moderate HIE, commonly includes seizures, it presents lethargy, hypotonia (typically a differential tone between upper and lower limbs with the arms being more hypotonic than the legs) and predominantly parasympathetic responses. The third stage, severe HIE, presents comatose infants with severe hypotonia, prolonged seizures, and absent primitive reflexes. These infants mostly need ventilatory support due to respiratory failure. According to Roberton's Textbook of Neonatology, the risk of death or severe handicap according to grade of HIE is 1.6% for mild HIE, 24% for moderate and 78% for severe HIE (11).

1.1.5 Mechanisms

Several mechanisms are involved in the brain injury caused by perinatal asphyxia (5, 12-14). A simplified schematic representation of the mechanisms involved is given in figure 1.

The damage is inflicted in two stages; the initial and the secondary phase. The initial phase is characterized by energy failure and a decrease in cerebral microcirculation. This leads to initiation of free radical production by the mitochondrial redox chain, and to a depolarization of neuronal cells with influx of Na^+, Ca^{2+} and Cl^- ions, and water, which ultimately generates cytotoxic edema (14, 15). The massive increase in free cytosolic calcium concentration is referred to as 'calcium overload', and has been shown to activate enzymes that degrade phospholipids, proteins, and deoxyribonucleic acid (16, 17). The membrane depolarization also results in the release of glutamate into the extracellular space. Energy dependent reuptake mechanisms become compromised, and glutamate accumulates to excitotoxic levels, overactivating the N-methyl-D-aspartate (NMDA) receptors which again lead to influx of Na^+ and Ca^{2+} (5, 15, 16, 18). The combination of energy failure, acidosis, calcium overload, glutamate release, free radical production, and lipid peroxidation ultimately leads to cell death (5). Between the initial and second phase of injury there is a free interval. In this interval, in cerebral tissue capable of recovery, the membranes are repolarized, and the energy metabolism is restored rapidly (5, 16, 17).

The second phase of injury occurs from approx 6 to 48 hours after the initial incidence, and is characterized by a secondary energy failure (19). In the second phase glutamate is believed to play a larger role than in the initial phase, at least in global asphyxia (20). Glutamate excitotoxicity is seen as one of the major mechanisms for neuronal death after perinatal asphyxia (14, 15). Other major events in the second phase of injury are free radical production and release of nitric oxide (17, 20, 21).

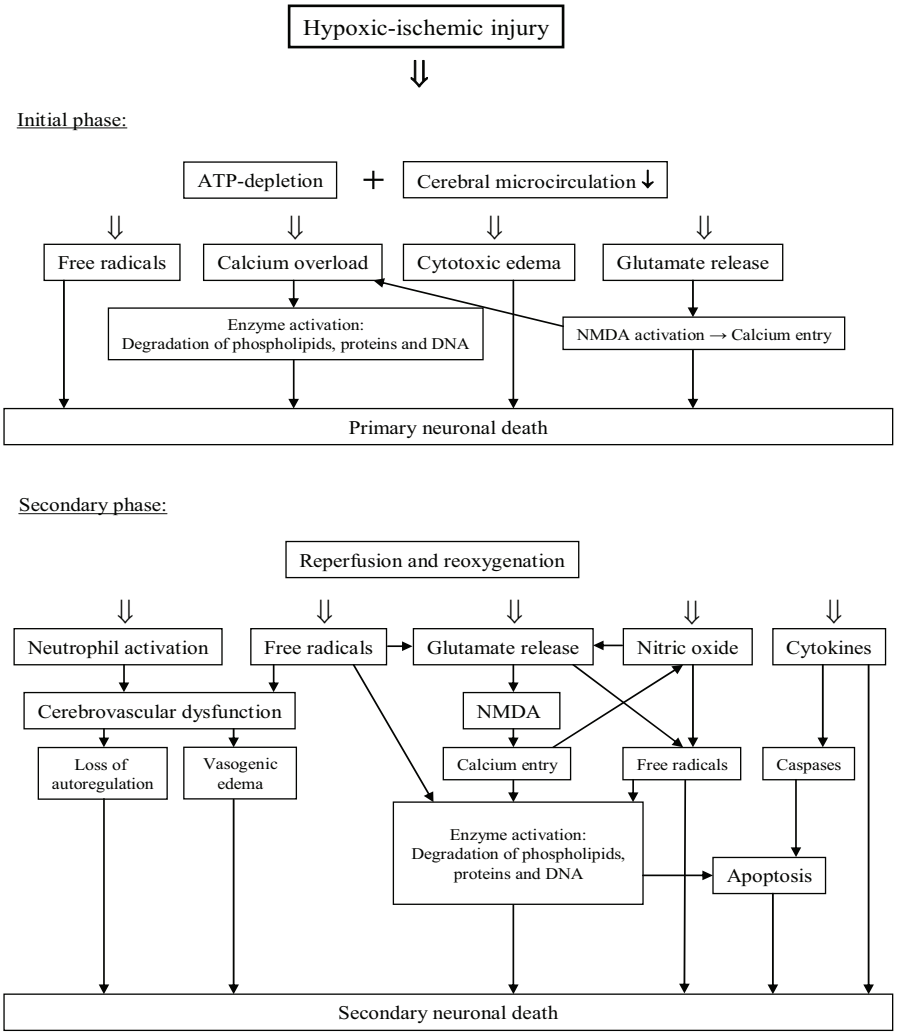


Figure 1. Simplified schematic representation of the mechanisms involved in HI injury.

The immature brain is especially vulnerable to oxidative damage due to high concentrations of unsaturated fatty acids, high rate of oxygen consumption, low concentration of antioxidants, and high availability of free iron for direct production of free radicals through the Fenton reaction. It has been shown that free iron increases in hypoxic-ischemic brain injury, and that increased free iron in grey matter persists for several weeks after the hypoxic-ischemic event (15, 21). Other mechanisms that contribute to the damage seen in perinatal asphyxia are apoptosis, necrosis, and inflammatory reactions with release of cytokines and chemokines (12, 13, 17). All these different mechanisms are linked together, and mitochondria play crucial roles in both the activation of apoptosis and the production of free radicals (21).

Free radicals

Free radicals are highly reactive atoms or molecules that contain one or more unpaired electrons. They can function as either reducing or oxidizing agents by donating or removing electrons from other molecules. The most important source of free radicals is the mitochondrial respiratory chain. Other sources are leucocytes, the hypoxanthine-xanthine oxidase system, and oxidation of arachidonic acid and catecholamines (22, 23). Free radicals are potentially harmful to cellular components, but normally exist in an equilibrium with innate cellular antioxidants, and are essential for fundamental cellular reactions and cell-cycle regulation (24). When biological processes lead to an increased free radical production, disturbing this equilibrium, it can result in oxidative damage to proteins, lipids, and DNA. Free radicals are part of the triggering of excitotoxicity and apoptosis (21).

Apoptosis and Necrosis

Apoptosis is an essential mechanism for maintaining homeostasis during development, and is often referred to as programmed/physiological cell death. It is an energy demanding process, and presents characteristic morphological changes such as condensation and fragmentation of the nucleus. There is no leakage of cytosol components and no inflammatory response. This is in contrast to cell death

caused by necrosis, which is induced by lack of energy. In necrosis the cells fail to maintain the normal electrolyte balance and ATP production, they swell and rupture. Cytosol components leak out into the surroundings and cause an inflammatory response. In neonatal hypoxia-ischemia it has been shown that both apoptosis and necrosis contribute to the subsequent damage (13, 25), and it has been found that the pathogenesis of hypoxic-ischemic brain damage is shifting from apoptosis to necrosis during brain development (26).

Mitochondria are key regulators in the process of cell death through their capacity to release a number of pro-apoptotic factors from their intermembrane space, such as cytochrome c, caspase-2 and -9, and apoptosis-inducing factor (AIF).

Caspases are a unique family of proteases that play an important role in the initiation and execution of apoptosis, with Caspase-3 acting as the key executioner (27). AIF triggers apoptosis in a caspase-independent manner, and can also induce caspase-activation (28, 29). There are at least three different pathways that lead to the execution of apoptosis: one caspase-independent pathway, involving AIF; one intrinsic pathway with apoptosome formation and caspase-9 cleavage, and one extrinsic pathway with binding of the Fas-ligand to its receptor and subsequent caspase-8 cleavage. The two latter both subsequently lead to caspase-3 activation, whereas AIF can induce caspase-activation by triggering the release of mitochondrial cytochrome c (18, 28, 29).

1.1.6 Distribution of damage

In mature infants the areas most often affected (i.e. most sensitive to hypoxic injury) are the cerebral cortex, hippocampus, cerebellum (purkinje cells), and the anterior horns of the spinal cord. Neuronal injury to basal ganglia is usually combined with injury to the thalamus, and is seen in approximately two thirds of asphyxiated term infants (1, 11). The distribution of white matter damage in the term infant is predominantly subcortical due to the distribution of the vascular

supply (30). The neurons show most damage while oligodendrocytes, astroglia and microglia mostly remain undamaged (20).

Several animal studies have investigated the distribution of damage in different kinds of insults. Clapp et al subjected fetal sheep to transient episodes of partial cord occlusion for 1 minute every 3 minutes for 2 hours, and found exclusively white matter damage (31); whereas Myers and coworkers show in their primate model that hypoxia without acidosis causes white matter damage, while severe acidosis during the hypoxic insult causes basal ganglia damage (32).

Periventricular leucomalacia is seen mainly in the immature/premature newborn, and is characterized by damage to the white matter dorsal and lateral to the lateral ventricle. It is increasingly rare after the 32^{nd} week of gestation (20).

The distribution of white matter damage in the mature and premature newborn is illustrated in figure 2.

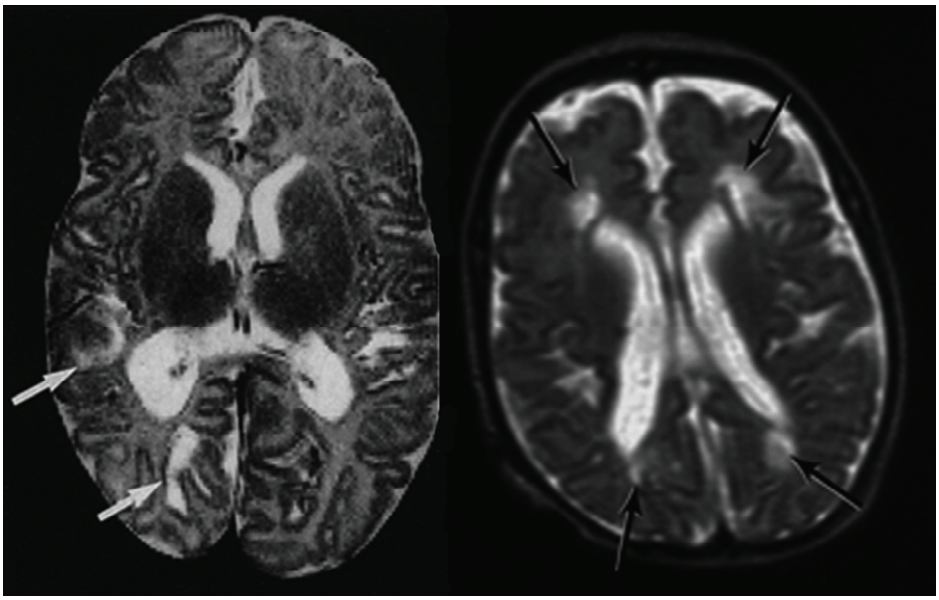


Figure 2. MRI images illustrating damage to subcortical white matter with cystic lesions in the left image (white arrows, from Baenziger et al 1993 (33)); and damage to the periventricular white matter in the right image (black arrows, from Counsell et al 2002 (34)).

1.1.7 Resuscitation and the use of oxygen

Guidelines for resuscitation of neonates were last published in 2005. The American Heart Association and the International Liason Committee on Resuscitation both offer guidelines as shown in the flow chart in figure 3. They include observations of respiration, heart rate (HR), and skin color (35, 36). 10% of all neonates require assistance to start breathing at birth, and 1% need further assistance (35).When performed properly, positive-pressure ventilation alone is effective for resuscitating the majority of apneic or bradycardic neonates. For about 80% mask ventilation alone is sufficient, whereas a small number require endotracheal intubation (37). If

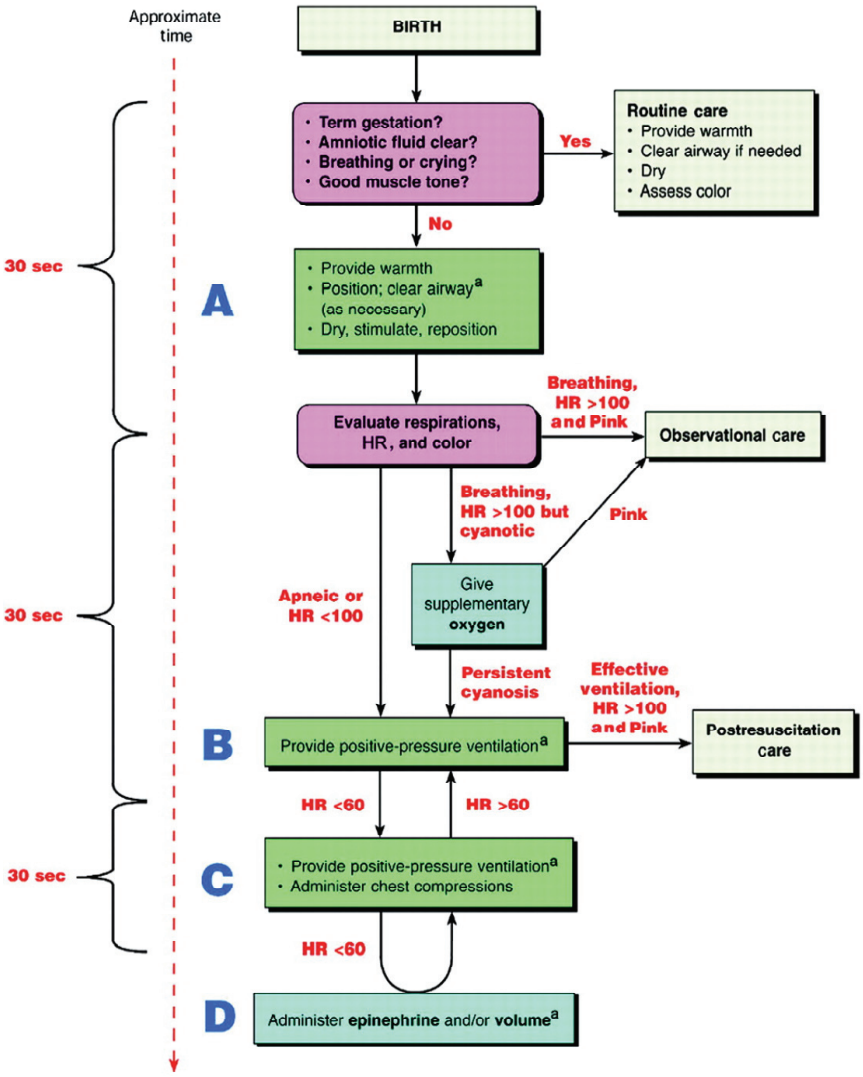
the heart rate remains < 60 bpm despite adequate ventilation for 30 sec, chest compressions are required, and should be carried out at a 3 : 1 ratio, with 90 compressions and 30 breaths per minute. Drug therapy with adrenaline (endotracheal up to 0.1 mg/kg, intravenous 0.01 – 0.03 mg/kg; iv administration being the preferred route of delivery (38)), and volume therapy (isotonic saline 10 ml/kg) should be considered if the HR does not respond after 30 sec of adequate ventilation and compression (35).

Regarding oxygen the guidelines differ slightly: ILCOR does not specify the concentration of oxygen to be used at initiation of resuscitation. They do however state that there is no evidence to support or refute a change in the oxygen concentration that was initiated, once adequate ventilation is established, and recommend that supplementary oxygen should be considered for infants with persistent central cyanosis (36). The American Heart Association recommends supplementary oxygen to be administered whenever positive-pressure ventilation is indicated, but they do open for the use of room-air if supplementary oxygen is not available (35).

The concept of hypoxia-reoxygenation injury through oxygen free radicals was introduced by Saugstad and Aasen in 1980 (39). Since then, the use of oxygen for resuscitation has been subject to extensive research. It has been demonstrated that room air is as efficient as 100% oxygen for neonatal resuscitation (40-42), and that 100% oxygen has possible detrimental effects in clinical and experimental settings (43-46). Both a Cochrane database systematic review and a meta-analysis have concluded that there might be insufficient evidence to recommend room air over 100% oxygen, or vice versa, but that a significant reduction in mortality in infants resuscitated with room air has been shown, and no evidence of harm demonstrated (47, 48). The trend worldwide is currently to reduce the oxygen concentrations used at resuscitation – the optimal concentration has however not been established.

Figure 3.

Neonatal flow algorithm



HR indicates heart rate (shown in bpm). a Endotracheal intubation may be considered at several steps.

1.1.8 Therapy and intervention strategies

To achieve satisfactory management of the asphyxiated newborn it is crucial to identify the infant at risk for evolving injury, to give supportive care to facilitate adequate perfusion and nutrients to the brain, to maintain glucose homeostasis, and to consider interventions to interfere with the process of ongoing brain injury (49).

Supportive care includes ventilation (avoiding hypocapnia), blood pressure support, seizure treatment, fluid management and avoidance of hypoglycemia (49). The supportive strategies are often based more on empiricism than evidence. In fluid management, a strategy widely practiced is fluid restriction. This is however poorly studied; a Cochrane review from 2005 on this subject concluded that no studies could be included, and that one could only recommend fluid restriction to the seriously injured newborns with renal failure (50). Regarding the use of Dopamine in hypotensive newborns subjected to perinatal asphyxia, a Cochrane review from 2002 concluded that only one study could be included in the review, and that it was thus impossible to make any recommendations (51). A Cochrane review published in 2001, and updated in 2007, on the use of anticonvulsants, concluded that there was not enough evidence to recommend the use of anticonvulsants for the prevention of severe neurodevelopment disability or to reduce mortality (52).

Due to the fact that the secondary energy failure occurs from 6 to 48 hours after the initial event, the therapeutic window is estimated to be between 2 and 6 hours after the insult (5, 22). Strategies aimed at reducing the extent of secondary brain damage involve therapeutic hypothermia, excitatory amino acid antagonists (xenon gas, magnesium sulfate), free radical scavengers (allopurinol, iron chelating agents), erythropoietin, calcium channel blockers (nicardipine) and the brain-derived neurotrophic factor (BDNF) (6, 49, 53, 54). In the following paragraphs we summarize some of the features of these interventional strategies.

Therapeutic hypothermia

All the mechanisms for the neuroprotective effect of therapeutic hypothermia are not clear, but it is known that mild hypothermia (reduction of core body temperature by 3˚C) ameliorates the pathophysiological processes following asphyxia (55). Hypothermia reduces the release of excitatory amino acids and free radicals, reduces production of NO and leukotrienes, increases levels of IL-10, reduces apoptosis, prevents blood-brain barrier disruption and brain edema, and reduces the cerebral metabolic rate. It has been found to reduce damage in cortex, thalamus and hippocampus (49, 56-58).

Therapeutic hypothermia is being assessed in several randomized trials in asphyxiated neonates, and so far the results are promising for the moderately asphyxiated neonate (59, 60). Only two reasonably large randomized clinical trials have so far reported 18 months follow-up data (61-63), presenting a significant reduction in death or disability. Regarding adverse effects, there is a risk of sepsis, thrombocytopenia, and arrhythmia, but so far no serious adverse effects have been reported in the conducted trials (56). Clinicians are now advised to follow guidelines from the conducted, or ongoing trials, if implementing hypothermia in clinical practice (60).

Excitatory amino acid /Glutamate receptor antagonists

Xenon gas

Xenon is a nontoxic anesthetic gas. It reduces neurotransmitter release and antagonizes glutamate receptors (N-methyl-D-aspartate (NMDA)-subtype), and thus has neuroprotective effects (64). It also has an effect on the pathways involved in apoptosis, and seems to have antiapoptotic abilities (65). Xenon is an expensive drug, but has the advantage of being an established medication literally free from side-effects. More studies are currently being conducted (65).

Magnesium sulfate

Magnesium sulfate is an antagonist to the glutamate receptors (NMDA), and blocks the neuronal influx of calcium (49, 66). It is used in perinatal medicine as a tocolyticum, and to prevent convulsions in preeclampsia. In this setting positive effects on outcome for premature infants have been found, which has lead to magnesium being assessed as a possible neuroprotective agent in perinatal asphyxia (67). Several studies have been conducted, both on animals and on neonates, with results showing both beneficial and non-beneficial effects (49, 67-69). Ichiba et al studied the use of magnesium sulfate in asphyxiated newborns, finding positive effects of a low dose over three days, both on short- and long-term follow up (70, 71). Groenendaal et al however, had to abort their randomized trial due to marked hypotension (with a somewhat higher dose than Ichiba et al) (72). In the last trial from Ichiba et al they report respiratory failure and severe muscular hypotoni in all the treated neonates after infusion of magnesium. They avoided the hypotension problems by simultaneously infusing Dopamine (71). As stated by Perlman in his review of intervention strategies; "further research is necessary to determine the potential neuroprotective role of magnesium" (49).

Free radical scavengers

Allopurinol

Allopurinol is a xanthine-oxidase inhibitor and free radical scavenger, and it has also been shown to have iron chelating abilities (73, 74). Van Bel and coworkers have shown positive effects on asphyxiated newborns (73), but the mentioned study had few included patients and was thus unable to show differences regarding death or neurological abnormality (6). A study by Benders et al (75) showed no improved short term outcome after severe birth asphyxia (Allopurinol given four hours after delivery), whereas a recent publication by Gunes et al (76) report improved neurologic and neurodevelopmental long-term outcome (12 months or more of age) when Allopurinol was given within two hours after delivery. Gunes et al do not provide information about the severity of the asphyxia in the follow-up data. Summarized there is not enough evidence to recommend clinical use of Allopurinol

for perinatal asphyxia at the present time. Maternal administration of Allopurinol in cases of fetal distress is currently being investigated (Van Bel and coworkers, presented at the 4th 'Europe Against Infant Brain Injury' (EURAIBI) meeting in Siena, Italy, April 2008).

Iron chelating substances – Deferoxamine

Non-protein bound iron (NPBI) is known to be liable to catalyze the formation of the hydroxyl radical through the Fenton reaction (77), and is thus thought to be a contributor to the damage seen in perinatal asphyxia. Deferoxamine is a well-known iron chelator, and research on animal models has shown that deferoxamine significantly lowers levels of NPBI in plasma and cerebral cortex. It has however also shown negative circulatory effects on newborn, preterm baboons (53, 74, 78). Due to these findings caution is warranted with the use of this substance in newborn humans (53).

Erythropoietin

Erythropoietin (Epo)/recombinant erythropoietin (rEPO) has been subject to extensive research in animal models of experimental brain injury over the last decade. It has been shown to have neuroprotective effects, and modulates a wide range of processes, including progenitor stem cell development, cellular integrity, and angiogenesis (79). Epo has anti-inflammatory, antiapoptotic and neurotrophic abilities (80). In the neonatal brain Epo is released by astrocytes and triggers the release of dopamine, promotes neurogenesis and vasculogenesis, and stimulates glial proliferation (81). Trials are currently being performed to assess the most appropriate dosage in neonates at risk for hypoxic brain damage/developmental problems (SE Juul 'A phase I/II trial of high dose erythropoietin in extremely low birth weight infants: pharmacokinetics and safety'. Oral presentation at the 48th annual meeting of the European Society for Pediatric Research, Prague, Czech Republic Oct. 6th-8th 2007). So far it seems to be well tolerated, and follow-up studies in rodents have shown no long-term negative effects (80).

Calcium channel blockers

The massive increase in free cytosolic calcium concentration, the so-called 'calcium overload', that is seen in the initial phase of perinatal asphyxia, has been shown to activate enzymes that degrade phospholipids, proteins and DNA (16, 17). This mechanism has lead to the hypothesis that calcium channel blockers would reduce the damage found in hypoxic-ischemic brain injury (66), and experimental models have shown positive effects (82, 83). However Levene et al published a study in 1990 on four infants with perinatal asphyxia, treated with the calcium channel blocker Nicardipine. The treatment was associated with clinically important hypotension (84). Following this, the recommendation has been to avoid calcium channel blockers in neonates and young infants because of significant adverse cardiovascular effects (49).

Brain-Derived Neurotrophic Factor (BDNF)

Neurotrophic factors are believed to play important roles in regulating neuronal connectivity in the developing central nervous system. BDNF is a neurotrophic factor highly expressed in the developing brain. It supports the survival and maintenance of specific populations of neurons, both in the peripheral and central nervous system (85, 86). Cheng et al (87) have shown a marked age-dependent neuroprotection by BDNF in rats. In neonatal rats they found a significant protection of brain tissue loss both when BDNF was given as pretreatment, and when it was given after the insult. The highest significance was found for the hippocampus. For adult animals however, no neuroprotection was shown. Similar findings of exogenous BDNF have also been demonstrated by others (88). BDNF has been found to have antiapoptotic abilities. It blocks activation of caspase-3, and decreases the up-regulation of other apoptotic proteins (phosphorylated c-Jun, cytochrome c) (89, 90). BDNF does however not cross the blood brain barrier (BBB), and must be given intracerebrally/intraventricular (91). This limits the use in clinical contexts. Agents that increase levels of BDNF could however be useful.

1.2 Nicotine

1.2.1 Historic data and general effects of nicotine

Tobacco was introduced in Europe from the Americas in the late 15th and early 16th centuries by sailors returning to various ports in Europe. Nicotine is named after the tobacco plant Nicotiana tabacum, which in turn was named after Jean Nicot, a French ambassador to Brazil. He introduced tobacco into the court of Catherine de Medicis in 1560, and promoted its medical use. The first empiric studies of nicotine were conducted in the 1950's (92, 93). Nicotine has mood-altering effects. It stimulates the release of several chemical messengers including acetylcholine, noradrenaline (norepinephrine), adrenaline (epinephrine), vasopressin, arginine, dopamine and beta-endorphin. Dopamine and glutamate are key neurotransmitters in the brain regarding nicotine's ability to induce dependency (94).

1.2.2 Negative effects of nicotine in the pre- and postnatal period

Smoking during pregnancy has long been known to cause adverse effects on the fetus, and nicotine is believed to be the main agent for this. Animal studies on rodents and monkeys have shown that nicotine, in doses comparable to moderate smoking, up-regulates nicotinergic acetylcholine receptors (nAChRs) in the brain, and thus has an impact on brain development since these receptors are involved in cell replication and differentiation in the fetus (95-97). A study on human neonatal brain tissue from aborted fetuses (<12 weeks) illustrates comparable effects of nicotine on neonatal human nAChRs (98). Ernst et al conclude in their review from 2001 that a dose-dependent relationship between maternal smoking and low birth weight and spontaneous abortion has been shown, and that there are indications of impaired neurodevelopment and possible higher risk for psychiatric problems and substance abuse after prenatal exposure to nicotine (99). It has been proposed that fetal exposure to nicotine activates apoptosis, measured on increase in c-fos mRNA levels in neonatal rodents (c-fos is a nuclear transcription factor elevated in apoptosis and cell injury) (100). A study on ante- and postnatal administered

nicotine in piglets has showed increase in apoptosis measured on TUNEL staining and caspase-3 (101). A strong correlation has been found between maternal smoking, both pre- and postnatal, and sudden infant death syndrome (SIDS) (102-105), and animal studies on both rodents and piglets have found nicotine to be responsible for this (106-109). Studies have also shown a correlation between maternal smoking and the development of asthma and respiratory symptoms in childhood, as summarized in two reviews by Cook et al (110, 111), and shown in a multi-centre study by Moshammer et al (112). Maternal nicotine exposure has been found to have an effect on lung surfactant system in newborn rats, suggesting that this could be of importance in the pathogenesis of impaired lung function in children exposed to intrauterine nicotine (113).

1.2.3 Positive effects of nicotine

Although the main focus on nicotine has been on negative effects, it has also been found to have several positive effects.

Epidemiological studies have shown decreased incidence of Parkinsons disease, Alzheimers disease, and ulcerative colitis in smokers (114-116). Nicotine is thought to be the main contributor to these effects, and several animal and *in vitro* studies have been conducted to find the mechanisms by which these effects are carried out (117-121).

Most studies on neuroprotective effects of nicotine look at nicotine administration prior to excitotoxic cell injury, inflammation, and hypoxia, but there are also a few looking into the effects of nicotine administered after an incidence (119, 122-128). This is the most interesting approach from a neonatal point of view, since there is seldom time or opportunity, nor a wish, to treat neonates at risk of perinatal asphyxia whilst intrauterine. Nicotine has been, and is still being, investigated as a possible neuroprotective, antiinflammatory and antiapoptotic agent in several settings. The mechanisms by which these effects are believed to be carried out are discussed below:

Nicotine and nAChR

nAChRs are a diverse family of ligand-gated ion channels, and binding of nicotine to their extracellular binding sites leads to influx of sodium and calcium ions (129). They are involved in a number of processes in the CNS. In neuronal development and survival they exert a trophic role, and the α7 subunit is implicated in several cellular processes like sensory perception, pain perception, body temperature regulation, neuroprotection, learning, and memory (130). Neuronal nAChRs are highly concentrated in the hippocampus, thalamus, and cortex. They play a role in enhancement of cognitive functions in the hippocampus and the cerebral cortex, in neuronal development in the sensory cortex, and in reward in the mesocorticolimbic system (131).

nAChRs are considered the main binding site for nicotine, and extensive research has looked at nicotine's binding to these, and the subsequent effects. The nicotine induced calcium influx after binding to the nAChR's, decreases the ability of glutamate (and other excitotoxic agents) to increase calcium levels (132). The nAChR consist of several subunits, and research has found that nicotine's effects on apoptosis and inflammation are carried out mainly through binding on the α7 and α4β2 subunits (123, 124, 127, 133-140). This has been shown by using substances blocking these receptors, and subsequently antagonizing the mentioned effects.

Nicotine and glutamate

Glutamate release is seen as a part of the reward system, and glutamatergic neurotransmission is involved in the dependency-producing effects of nicotine (141, 142). The effect of nicotine on glutamate has been investigated by Meshul et al (119) in an experimental rodent model. They show that the effect is dose- and time-dependent, with higher doses, and long term treatment, inducing the release of glutamate; and smaller doses, and short term/subchronic (7 days) treatment, causing reduction in glutamate levels. Nicotine influences the release of dopamine in a time-dependent way, with short term treatment causing increase in dopamine levels, and long term causing decrease (122, 143, 144). Increased levels of extracellular

dopamine have been reported to result in decreased striatal glutamate release (145, 146). This might explain the effects of nicotine on glutamate. Furthermore, nicotine protects against glutamate induced neurotoxicity. This has been found to be mediated by nAChR's, as shown by Sun et al (138), and Dajas-Bailador et al (124).

Nicotine and calcium

Nicotine has been shown to modulate glutamate induced increases in intracellular calcium (124, 147), and to reduce intracellular calcium concentration (138). This could be explained by the fact that nicotine has been found to activate calcineurin, a calcium dependent phosphatase, thus down-regulating the activity of L-type calcium channels (147). This might be one of the mechanisms by which nicotine exerts its neuroprotective effects.

Nicotine and mitochondria

Cormier et al (148, 149) have done experiments both *in vitro* and *in vivo* showing that nicotine has direct effect on mitochondria. They have found that nicotine binds directly to the complex I in the respiratory chain in the mitochondriae, antagonizing the $NADH/H^+$ binding. This induces a decrease of free radical generation. Nicotine does not bind to the nAChR on the mitochondria, meaning that the mitochondria related effects of nicotine are nAChR independent.

Further nicotine has been shown to inhibit mitochondrial swelling and cytochrome c release due to inhibition of the mitochondria permeability transition pore (mPTP). mPTP is involved in apoptosis through the release of cytochrome c, cytochrome c is a critical factor for triggering apoptosis through activation of the caspase cascade (121, 150). Findings by Xie et al support the assumption that there is a receptor-independent neuroprotective effect of nicotine (121). These effects on mitochondriae could contribute to nicotine's anti-inflammatory and anti-apoptotic effects.

Nicotine and free iron/the Fenton reaction

The Fenton reaction produces oxygen radical species in the presence of free iron, and contributes to the formation of free radicals in hypoxic-ischemic brain injury of the neonate (77, 151-153).

The Fenton reaction:

(1) $Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH\cdot + OH^-$

(2) $Fe^{3+} + H_2O_2 \rightarrow Fe^{2+} + OOH\cdot + H^+$

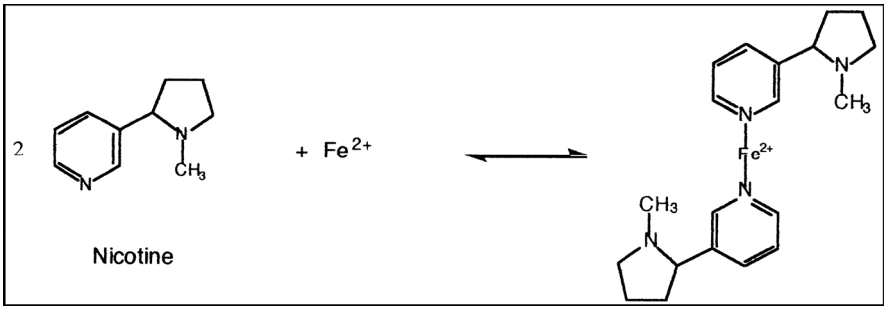


Figure 4. Nicotine forms complexes with free iron (Fe^{2+}), here shown with binding to the pyridine nitrogen.

Research on cellular level has shown that nicotine is capable of chelating free iron (154), and the findings of nicotine blocking the Fenton reaction is believed to be mainly due to this (120).

In addition, the inhibitory effect of nicotine on the Fenton reaction leads to less oxidation of dopamine to the neurotoxic 6-hydroxydopamine (6-OHDA), and nicotine also inhibits the autoxidation of 6-OHDA, which leads to production of OH^-, which again can produce H_2O_2, and trigger the Fenton reaction (117, 120). This is thought to be an important aspect of nicotine's positive effects on Parkinson's disease.

Further, nicotine has local effects on noradrenaline release (155), and Traver et al have shown that noradrenaline leads to a decreased concentration of ROS produced by the Fenton reaction (156). The inhibition of the Fenton reaction contributes to nicotine's ability to reduce the concentration of free radicals in e.g. hypoxic-ischemic brain injury.

Nicotine and neurotrophic factors/BDNF

Nicotine can increase the levels of some neurotrophic factors, like the nerve growth factor (NGF), the basic fibroblastic growth factor (FGF-2), and the brain-derived neurotrophic factor (BDNF). This effect is especially prominent in the hippocampus (118, 135, 157-159). Garrido et al show that nicotine treatment significantly upregulates NGF-expression (157). They have also demonstrated that in cultured spinal cord neurons exposed to arachidonic acid, nicotine pretreatment markedly protected against decrease in BDNF mRNA levels. However, nicotine did not affect the baseline BDNF mRNA expression (135). This indicates that nicotine exposure could prevent the decrease in BDNF levels that has been shown after hypoxia and inflammation in cell cultures. Nicotine's effect on neurotrophic factors might be a part of the mechanisms by which nicotine carries out its anti-apoptotic effects.

Nicotine and inflammation

In recent years the expression 'nicotinic anti-inflammatory pathway', or the 'cholinergic anti-inflammatory pathway' has emerged, indicating that the vagus nerve can modulate the immune response and control inflammation dependent on the α7nAChRs (160, 161). Nicotine has been proven more efficacious than acetylcholine at inhibiting pro-inflammatory cytokines like IL-1, IL-6, TNFα and HMGB1 (high-mobility group box 1). The impact on TNFα is carried out mainly through effect on the macrophages, which express α7nAChRs (128, 162). It has been shown that the effect of nicotine on the mentioned cytokines is a post-transcriptional one, since there is no modulation of intracellular mRNA levels (128, 163). Nicotine also has an impact on the secretion of pro-inflammatory cytokines by

inhibiting the NF-κB (nuclear factor- κB) pathway, probably by preserving cytoplasmatic levels of the inhibitor of NF-κB (the IκBα inhibitor) (125, 160, 164).

Regarding clinical use, ulcerative colitis is the only condition for which controlled trials have provided evidence of the therapeutic potential of nicotine (165). The therapeutic use of nicotine has been suggested for the treatment of several conditions like Tourette's syndrome, Parkinsons disease, and Crohn's disease (160). In sepsis nicotine has shown effect by significantly improving survival in an experimental setting, the main effect being binding to macrophages and inhibiting release of HMGB1 (128, 161). In an experimental study on renal ischemia/reperfusion injury nicotine has shown beneficial effects by inhibiting neutrophil infiltration, reducing TNFα and HMGB1, and has also presented anti-apoptotic abilities (166). Wittebole et al carried out an experiment on human subjects, studying the response to bacterial endotoxin or lipopolysaccharide (LPS) after pretreatment with nicotine or placebo (167). The results showed that nicotine pretreatment gave attenuated febrile response to LPS, and increased circulating IL-10 and cortisol levels.

In summary it has been shown that nicotine has an apparent impact on inflammatory responses, both peripherally and in the central nervous system.

Nicotine and apoptosis

Nicotine has been shown to act on the nAChRs and inhibit caspase activation (caspases 3, 7, 8 and 9), and it acts directly on mitochondria preventing the release of cytochrome c which is a caspase activator (121, 127, 136, 150, 166, 168). Sun et al (138) presented increase of the anti-apoptotic protein bcl-2, and decrease of the pro-apoptotic protein bax after 24 hours of nicotine treatment prior to hypoxia; this was proposed to be mediated through binding on nAChRs. Nicotine's effect on neurotrophic factors is also believed to be one of the mechanisms by which nicotine exerts its anti-apoptotic effects (135, 158).

Nicotine and the sympathetic nervous system

Nicotine is a known activator of the sympathetic nervous system. It increases the central nervous system sympathetic outflow; leads to catecholamine release from the adrenal medulla, from tissue stores, through stimulation of autonomic ganglia and peripheral chemoreceptors, and through local release from vascular nerve endings (94, 169). It increases systemic adrenaline (epinephrine) and noradrenaline (norepinephrine) in a complex dose-dependent manner (170, 171). Clinically this is observed as increased heart rate, blood pressure, and coronary blood flow (94). Very low doses are thought to act mainly on the CNS, whereas higher doses act more on the peripheral sympathetic nervous system. Extremely high doses of nicotine, however, induce peripheral ganglionic blockade, vagal afferent-nerve stimulation, and has direct depressor effects. Thus hypotension and slowing of the heart rate occurs when nicotine is administered in extreme doses (172). Several investigators have found that nicotine has local effects on noradrenaline release in cerebrum (155, 173, 174). This could contribute to the neuroprotective effects of low doses of nicotine (156).

2. Aims of the study

Perinatal asphyxia is a major cause of mortality and morbidity, especially in the developing world. There is an ongoing search for interventional strategies that are safe, easy to use, cost-efficient, and most importantly – effective (5). Further, it is still debated what oxygen concentration should be used for resuscitation, although there is strong evidence against the use of 100% oxygen (175). We wanted to investigate the following issues:

1. What would the effects of resuscitation with room air versus 100% oxygen be on the newborn brain after nicotine exposure? We chose to focus on morphological changes in cerebellum, striatum and cortex, hypothesizing that resuscitation with 21% oxygen in nicotine exposed animals would cause less damage to the neurons compared with 100% oxygen (paper I).

2. What effect would pretreatment with nicotine have on the ability to endure hypoxia? We hypothesized that nicotine would have an effect on how long the piglets endured hypoxia; and that despite nicotine's neuroprotective effects it would not be able to counteract 100% oxygen's potentially harmful effect on the hypoxic piglet brain (paper I).

3. Could some of nicotine's neuroprotective effects be explained by systemic activation of the sympathetic nervous system? We hypothesized that nicotine in a low and moderate dose would not have any effect on plasma catecholamine levels (paper II).

4. Would post-hypoxic treatment with nicotine in our model of neonatal hypoxic-ischemic brain damage have the same effects on free iron and glutamate as shown *in vitro* and in adult animal models, and thus indicate possible neuroprotective effects in asphyxiated neonates? (paper III)

5. How would nicotine affect BDNF and apoptosis in the hippocampus in the hypoxic newborn piglet when given after a hypoxic-ischemic insult? We hypothesized that nicotine would decrease the levels of AIF and caspase-3 mRNA expression, increase levels of BDNF mRNA-expression, and increase levels of BDNF-protein in the hippocampus. This would imply a possible neuroprotective effect of nicotine infusions in hypoxic brain damage in the neonate (paper IV).

3. Materials and Methods

3.1 The animal model

Research on perinatal asphyxia is dependent on good models, as it is difficult to study the pathophysiology of this condition in humans. Over the last approximately 40 – 50 years several models have been developed, involving non-human primates, puppies, immature rodents, lambs, and piglets (176). Much of our current understanding is based on studies in these models.

An important issue in animal research is the difference in susceptibility to different interventions and treatments between species. For catecholamine response after injection of nicotine it has been shown that most species react in a similar manner (with a dose-dependent increase in plasma catecholamine levels) , with the exception of the fetal lamb, which has no response to nicotine infusions regardless of the doses used (177). This illustrates one of the caveats in research on animal models.

The piglet model has advantages due to size and body weight, which matches that of a human newborn. This makes piglets easy to work with, and accessible to the same equipment as used in neonatal intensive care units. In addition, the anatomy and physiology of pigs are comparable to humans (178), and most importantly for the research on perinatal asphyxia – their brains show substantial similarities to the human brain. Brain growth and myelinization (179), brain maturation (180), and distribution of grey/white matter (181) are all comparable to that of human neonates. The degree of myelinization makes the newborn pig a better candidate for research on perinatal asphyxia than the non-human primate. Non-human primate newborns have substantially more mature brains at birth, with complete myelinization. This gives different distribution of damage in the primate model subjected to perinatal asphyxia (32, 181).

Compared to the human neonate, cerebral blood flow (CBF) in the newborn pig is elevated, and it has higher rates of cerebral metabolism (182). Studies aiming at finding the lower level of cerebral autoregulation (LLA) for newborn pigs have come up with values between 35 and 40 mmHg (183, 184).

Different ways of inducing asphyxia/hypoxia-ischemia have been developed in the newborn pig. There are occlusion-models with occlusion of the common carotid arteries combined with low FiO_2 concentrations that induces both global hypoxia and local ischemia (185, 186), and there are global hypoxia models with either constant or variable FiO_2 (187-189). The global hypoxia models are able to induce ischemia when MABP reaches levels below LLA for longer periods of time. The animals in the global hypoxia models are monitored in different ways, with the variable FiO_2 model using aEEG as an indicator of brain activity to regulate the amount of oxygen given, and the constant FiO_2 model using MABP and BE to monitor the animals. The only comparative study done seems to favor the variable FiO_2 model, finding that this gives the most predictable amount of injury (187).

A drawback with the model used in the current study is the fact that the animals are subjected to global hypoxia at an age of 12-36 hours, meaning that they have already to some extent adapted to extra-uterine life. Further, in the present work the animals were normocapnic during hypoxia, which is a weakness since arterial pCO_2 is a strong determinant of CBF (190), and hypercapnia in perinatal asphyxia may influence the outcome by affecting both the general and cerebral circulation. There is large inter-individual variability in the newborn piglet model, causing problems regarding sample size and statistical analyses.

3.2 Anesthesia

Research performed on animals should always have as a prime incentive to provide optimal conditions for the animals, eliminating sources of stress, pain and general

discomfort. Thus the studies conducted in the present work had to be done in general anesthesia. This might lead to drugs acting as confounders, but care is taken using the optimal drugs and dosages to minimize this. It is however one of the drawbacks of results obtained in animal research.

3.2.1 Procedure

The animals were weighed and handled when awake, using heated towels and calm surroundings to minimize stress. They were then given gas as an introductory anesthetic as shown in figure 5.

Figure 5. Administration of gas anesthetics.

In paper I the animals were given Halothane 4% (Fluthane ZENECA). In papers II-IV they were given Sevoflurane 5% (Sevorane, Abbott), reduced to 2% before an ear vein was cannulated. Halothane/Sevoflurane was then disconnected, and the piglets were given pentobarbital sodium 20 mg/kg and Fentanyl 50 µg/kg

intravenously as bolus injections. Anesthesia was maintained by a continuous infusion of Fentanyl (50µg/kg/h) and Midazolam (0.25mg/kg/h; IVAC P2000 infusion pump). The dept of anesthesia was monitored by response to painful stimuli elicited by pinching between the toes, in addition to standard monitoring of heart rate and blood pressure. When considered necessary, a bolus of Fentanyl (10 µg/kg) or Midazolam (1 mg/kg) was added. In paper III and IV the animals were given pancuronium bromide (0.1 mg/kg) to eliminate shivering that did not cease with additional anesthetics. Shivering is known to occur in piglets even if anesthesia is sufficiently deep, and could possibly have interfered with the experiment regarding cerebral oxygen consumption and the position of the microdialysis probes. A continuous i.v. infusion (saline 0.7% and glucose 1.25%, 10 ml/kg/h) was given throughout the experiments.

3.2.2 Halothane/Sevoflurane

In domestic large white pigs Halothane is known to have the ability to induce malignant hyperthermia and tetanus. It has however been shown that this effect is more prominent in older animals, and not as frequent in piglets (191). Further, Halothane depresses cardiovascular function and leads to decrease in blood pressure, heart rate, and CBF (191). These effects are time and dose dependent, and Halothane was only given for a few minutes in paper I. In the following papers we changed to Sevoflurane administration. Sevoflurane has been found to be a safe and effective choice of anesthetics for children down to the age of 1 month (192), and it has been used in piglets and other animal models both in veterinary praxis and in research (193, 194). It has a cardio-depressive effect, but apparently not as strong as Halothane. Sevoflurane induces a slightly faster onset of anesthesia than Halothane (194), and was only given for a short period of time.

3.2.3 Pentobarbital

Pentobarbital, a well-known barbiturate in animal research, was used for the induction of i.v. anesthesia, and for the final overdose. Barbiturates are potent

cardiac depressants, with dose-dependent effects (195), but are generally well tolerated in pigs (196). They reduce CBF, intracranial pressure and the cerebral metabolic rate of oxygen (197, 198). Animal and *in vitro* studies have shown neuroprotective effects of barbiturates against hypoxic-ischemic brain damage (199-201). The doses used in the current study were small, and were not expected to influence the results.

3.2.4 Fentanyl

Fentanyl was used for analgesia. Pigs have been shown to be relatively resistant to the effect of narcotic analgesics, and thus require larger concentrations of opiates than many other animal models and humans (202). This has been found explicitly for Fentanyl by Moon et al (203). Fentanyl can cause vasoconstriction of cerebral arterioles, possibly decreasing CBF (204, 205), and can increase cerebral fractional oxygen extraction (206). Further, it has been shown to be able to induce chest wall rigidity at high doses/rapid administration in humans (207). There appears to be no publications on this phenomenon in pigs, but it has been observed by our group. Care was taken during the present work to administer Fentanyl slowly, carefully monitoring the effect.

3.2.5 Midazolam

Midazolam as a sedative agent has proven effective in pig models, and has minimal effects on the cardiovascular system (208). It has however been demonstrated in a piglet model to increase cerebral fractional oxygen extraction – suggesting compromised cerebral perfusion and oxygenation (209).

3.2.6 Pancuronium

Pancuronium, a non-depolarizing muscle relaxant, has been investigated in a newborn piglet model by Easa et al. In their paper from 1993 they concluded that pancuronium administered to newborn piglets during normoxia, and during, or

after, hypoxia, while mechanically ventilated, does not alter the cardiovascular or pulmonary hemodynamic status (210).

3.2.7 Implications

Most of the anesthetics in the present work are capable of interfering with hemodynamics and cerebral blood flow. However, all the animals received the same drugs, so the anesthesia should have minor influence on the differences found between the groups.

3.3 Microdialysis

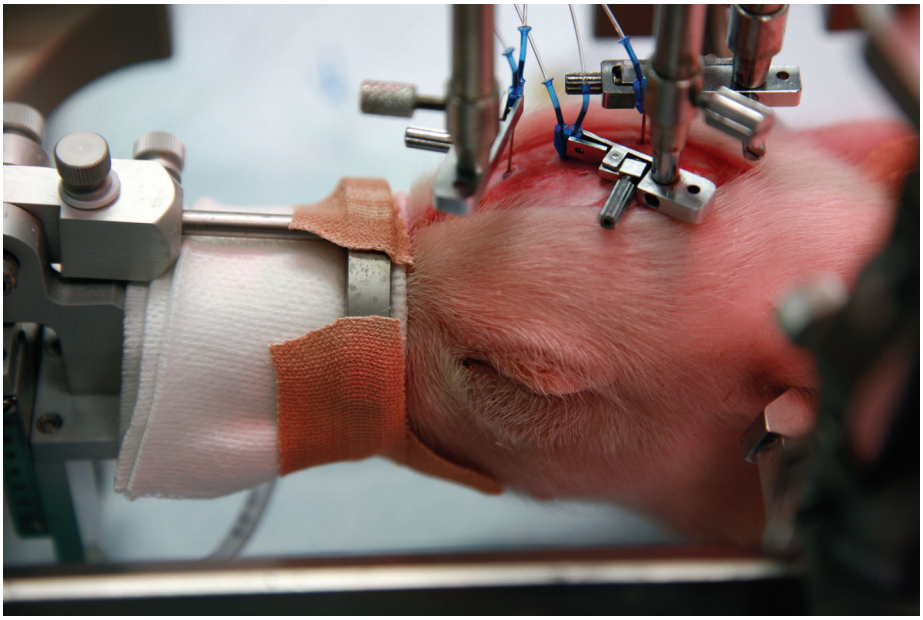


Figure 6. Newborn, anesthetized piglet in stereotactic frame with cerebral microdialysis probes.

Microdialysis has been used in research since the early 1970's, initially solely in neuroscience (211). Currently it is also being used clinically, in a number of different tissues and organs (212). It is used for sampling of drugs, metabolites, or endogenous substances from fluids or the interstitial cell fluid of selected tissues. The principle behind microdialysis is a simple diffusion of low-molecular-weight substances down a concentration gradient from the extracellular fluid compartment to the dialysis fluid compartment within the microdialysis probe (213). The probe is constantly perfused with a perfusion fluid that mimics the composition of the surrounding medium, preventing excessive migration of molecules into or out of the periprobe fluid due to osmotic differences.

The size of the molecules recovered is dependent on the 'cut-off' value of the probe, indicating the size of the holes on the dialysis membrane. It has been shown that only molecules about one fourth of the weight of the 'cut-off' value of the membrane are successfully recovered (212). In papers I and III we used a 'cut-off' value of 20 000 kDa.

The analyte recovery is further dependent on the temperature, concentration gradient, and flow rate over the membrane. In general low flow rates results in larger recoveries than high flow rates. Low flow rates being rates from 0.5 – 5 µl/min (212). We used a flow rate of 1 µl/min in our experiments. Analyte recovery can be either relative or absolute. Relative recovery is expressed in concentrations per volume; it describes the ratio between the concentrations in the dialysate to that in the periprobe fluid. Absolute recovery refers to the amount of compound in the dialysate per unit of time. Recovery will never be 'absolute' in the sense of directly reflecting the tissue/periprobe fluid concentration, it will always only be a fraction of the actual analyte concentration (212). It is however possible to determine recovery rate of the probes for the desired analyte prior to the experiment, giving the opportunity to calculate absolute values. This was not done in our experiments.

The introduction of the probe should be done slowly to prevent cellular damage and bleeding. The insertion will cause a disruption of the BBB and an injury-mediated

release of neurotransmitters in the local tissue, and at least one hour should be allowed after insertion to reach baseline conditions (214).

The analysis of microdialysate was done using the CMA 600 Microdialysis Analyzer (using enzymatic reagents and colorimetric measurements) for glycerol and glutamate, and non-protein bound iron was analyzed using spectrophotometry (using bathophenanthroline disulfonate (BPS) to chelate ferrous iron).

3.4 Pathology

Histopathologic visible ischemic cell change has been found to appear quite rapidly after ischemic brain damage, with microvacuolation of the neuronal cytoplasm being the first sign. These changes occur as early as 15 minutes after ischemia. The ischemic cell change is recognizable after 30-35 min, persisting up to 4 hours, with more pronounced damage being evident after 90 -150 minutes. Typically the watershed area of the cortex is injured first (215). The development of damage is an ongoing process for up to 24 hours after the insult. For severe damage the time-span for developing visible damage will be shorter than for less severe damage (181).

Tissue for histopathological evaluation is stained using hematoxylin and eosin (H&E), an established method for evaluating morphological changes in neuronal tissue. Cerebral necrosis is defined by the presence of vacuolated neuropil, shrunken neurons with pyknotic nuclei, and eosinophilic neurons. In cerebellum necrosis is defined by the presence of necrotic Purkinje cells with eosinophilic cytoplasm. We performed additional immunohistochemical staining with MAP-2 to confirm the areas of damage found with H&E staining.

MAP-2 (microtubule-associated protein 2) is an important cellular component of the neuronal cytoskeleton. It is a major component of all neurons and is highly localized to the somato-dendritic compartment. MAP-2 has been shown to regulate

the assembly and stability of neuronal microtubules, and has been suggested to help in regulating a balance between rigidity and plasticity in neuronal processes. It has been found to be a sensitive marker for ischemia in neurons and is down-regulated in this form of injury, with changes detectable as early as three minutes after ischemia (216, 217).

In paper I the animals were observed for 150 minutes after hypoxia/resuscitation, before receiving an overdose with pentobarbital. Tissue blocks from striatum, cortex and cerebellum were stained with H&E. The evaluation was done blinded, and damage was classified as present (+), or not present (-). MAP-2 immunohistochemistry was used to confirm areas of ischemic cell damage. Due to the short time of observation, the damage was classified as 'early necrosis'.

In paper IV the animals were observed for four hours after hypoxia, before receiving an overdose with pentobarbital. Tissue blocks from striatum, cortex and cerebellum were stained with H&E. The evaluation was done blinded, and damage to striatum and cortex was divided into five different categories: 0 = no damage found; 1 = ≤10% of the tissue damaged; 2 = 20-30% damaged; 3 = 40-60% damaged; and 4 = >75% of the tissue damaged. MAP-2 immunohistochemistry was used to confirm areas of ischemic cell damage. For the cerebellum the hypoxic/ischemic changes were defined by the presence of necrotic Purkinje cells with eosinophilic cytoplasm. In each case the number of eosinophilic Purkinje cells was counted in one section from the vermis of the cerebellum. Damage was classified as: 0 = no necrotic Purkinje cells; 1 = <50 cells; 2 = 50-150 cells; 3 = >150 cells.

The histopathological evaluations have the drawback of having been conducted not by a standardized machine, but by a human being. They were also done only by one pathologist, which could raise question regarding reproducibility. The MAP-2 staining contributed to confirm the findings on H&E staining, and the evaluations were done blinded, by an experienced pathologist. We thus believe that they are comparable, reproducible, and of good quality.

3.4.1 Immunohistochemistry

Immunohistochemistry is a combination of an immunoreaction identifying a specific protein/substance in a selected tissue, and the detection of that reaction using light microscopy. The principle is binding of an antibody to an antigen in the tissue-sample, either direct (using a labeled primary antibody) or indirect (with labeled secondary antibodies). The staining can be performed with immunoenzyme-techniques, or immunofluorescence. Immunofluorescence requires specialized microscopes for evaluation, and lack the easy comparison with the morphology in the tissue sample. For detection the antibody is either labeled with a fluorescent (Fluorescein, Rhodamine), or an enzyme that is linked with a chromogenic substrate to develop color (e.g. Avidin-biotin peroxidase (ABC) and Diaminobenzidine (DAB)).

For the fixation of the tissue both formalin and freezing can be adequate, but require different preparation before adding of the antibodies. Formalin fixation and paraffin embedding leads to 'masking' of the antigens, and 'demasking' can be achieved by different enzymatic or denaturing procedures that break up the protein meshwork. Procedures often used are: proteolytic enzymes, chemical denaturation, and boiling (microwave). (Reference: 'Methods in biomedical research', lecture by Per Brandtzæg (RH) august 2006 on 'Immunohistochemistry and immunocytochemistry'). The evaluation of immunohistochemistry is often done by computers with special software. We did not have such a machine when evaluating our results from paper IV, but the counting was done blinded, and followed a predefined setup. A drawback of working with antibodies is the variation in specificity of the antigen binding. Both the antibodies used in the present work were of satisfactory specificity. MAP-2 antibodies are highly specific, and have no known cross-reactions according to the manufacturer. The specificity of the BDNF antibody used in paper IV was 95%, with 5 % cross-reactivity with rhβ-NGF and rrβ-NGF.

3.5 Real time Polymerase Chain Reactions

The polymerase chain reaction (PCR) was invented by the 1993 Nobel laureate Kary B. Mullis in 1985. It uses a polymerase to catalyze the regeneration of DNA with a chain reaction that is repeated over and over to exponentially amplify the target DNA. This allows genetic material to go from scarce to abundant for analysis. Real time PCR amplifies a specific target sequence in a sample, monitoring the amplification process using fluorescent technology. The fluorescence signal increases in direct proportion to the amount of PCR product in a reaction during each cycle (in real time) as opposed to the conventional method where the product is detected at the end point. This increases the sensitivity of detection of the PCR product, leading to better accuracy and reproducibility (218). The major disadvantage to real time PCR is the requirement of expensive equipment and reagents; another drawback is that the method is relatively sensitive to minor variations in reaction components, thermal cycling conditions, and mispriming events during the early stages of the reaction. This can lead to large changes in the overall amount of amplified product. When handled correctly it is however a sensitive, reproducible and efficient method (219).

3.6 Catecholamine measurements

Catecholamines can be measured in urine, plasma, and in platelets (220). Urine-catecholamines are normally measured after collecting urine for a longer period of time, and is thus unsuitable for analyzes of changes happening over shorter time periods. Measurements of catecholamines in plasma is an established method, but because catecholamines in blood are easily degradable, it is required that the blood samples are collected in pre-treated containers (e.g. EGTA-gluthatione) kept on ice, and centrifuged immediately after sampling. There are different methods for

analyzing catecholamines in plasma (221). One of the well-established methods is high performance liquid-chromatography (HPLC). In paper II plasma noradrenaline (NA) and adrenaline (A) were determined by HPLC with a reverse phase column and glassy carbon electrochemical detector (Agilent Technologies, Colorado, USA), using a commercial kit (Chromsystems, München, Germany). The intra- and interassay variations were 3.9% and 10.8% for NA, and 13.3% and 14.8% for A respectively; the detection limit was 5.46pmol/l. Sources of error in this method are suboptimal storage of the vaccutainer containers and of the samples; and wrong treatment and optimizing of the samples. Another source of error is use of paracetamol, which can give a false positive NA value. Paracetamol was not used in the current study. Both the containers and the samples were treated as recommended (kept on ice and frozen immediately after centrifugation).

We are using pmol/l, whereas in the literature values are often referred as pg/ml. For results obtained by the same method (HPLC) this is easily converted by the following formulas:

Noradrenaline (MW 169.18 g/l): pg/ml · 5.9 = pmol/l (0.16918 pg/ml · 5.9 ≈ 1)

Adrenaline (MW 183.2 g/l): pg/ml · 5.4 = pmol/l (0.18320 pg/ml · 5.4 ≈ 1)

3.7 Measurements of nicotine concentrations

Nicotine analyzes are frequently used in smoking-related research, although the measurement of coitinine, the metabolite of nicotine, is more commonly used for assessment of tobacco-intake. We measured nicotine concentrations to assess our administered doses, in order to compare these to concentrations previously reported

and to concentrations seen in smokers (to compare with levels that are known to be non-toxic). Gas chromatography-mass spectrometry (GC-MS) is the most common way of measuring nicotine (222, 223).

Nicotine analyzes in the current work were done using gas chromatography-mass spectrometry (GC-MS), the analyzes were carried out at the Norwegian Institute of Public Health, Division of Forensic Toxicology and Drug Abuse. The lower limit of detection was 0.05 μM. The drawback of this method is the lack of standardization. It is not routinely performed, and is referred to as an experimental method. It has however been performed quite often in the Forensic Toxicology lab, and the results are therefore seen as reliable and reproducible.

4. Main results of the study

Paper I

Resuscitation with 21% or 100% oxygen in hypoxic nicotine pretreated newborn piglets. Possible neuroprotective effects of nicotine.

Animals pretreated with nicotine endured a significantly longer time of hypoxia before reaching defined endpoints (BE < -20 mmol/l and/or MABP ≤ 20 mmHg), compared to animals treated with saline prior to hypoxia (103.8 ± 28.2 min *vs.* 66.5 ± 19.5 min, p = 0.035). There was early cerebral necrosis (striatum and cortex combined) in 6 of 8 animals in the nicotine pretreated hypoxic group resuscitated with 100% oxygen, and 1 of 7 animals in the nicotine pretreated hypoxic group resuscitated with 21% oxygen (p=0.036, 95% CI 0.0 – 0.693). There was a significant difference between the two mentioned groups regarding striatal (0 of 7 *vs.* 5 of 8, p=0.026) early necrosis, but not for cortical (1 of 7 *vs.* 5 of 8, p=0.063) early necrosis.

We observed a decrease in early necrosis both when combining striatal and cortical necrosis, and for striatal necrosis alone in the animals resuscitated with room air compared to 100% oxygen; for cortical necrosis there was a trend towards less necrosis in the animals resuscitated with room air. These findings support other studies in the conclusion that 100% oxygen should not be used routinely for neonatal resuscitation. The results further suggest a possible neuroprotective effect of nicotine.

Paper II

Nicotine in a small to moderate dose does not cause a significant increase in plasma catecholamine levels in newborn piglets.

Newborn piglets with comparable levels of plasma catecholamines at baseline were randomized to three different groups, receiving nicotine-infusions (for 1 hour) with one of three concentrations of nicotine. Adrenaline/Epinephrine increased significantly in the group treated with 1000µg/kg/h nicotine ($p=0.019$), but not for the groups treated with 130 or 260µg/kg/h nicotine. There were no significant increases in noradrenaline/norepinephrine in either of the groups. Thus we find that nicotine in a small and a moderate dose does not generate a significant increase in plasma catecholamine levels, whereas a higher dose significantly increases plasma adrenaline values. This confirms the dose dependent effect nicotine is believed to have on the sympathetic nervous system. These results suggest that the positive effects of nicotine found in studies with nicotine administered in these small/moderate doses, can probably not be explained by the systemic release of catecholamines.

Paper III

Effects of nicotine infusion on striatal glutamate and cortical Non-Protein Bound Iron in hypoxic newborn piglets.

Microdialysis was used to sample dialysate from striatum and cortex at baseline, after hypoxia, and during the observation period. For striatal glutamate there was a significant rise from baseline to the end of hypoxia for all the animals ($p < 0.001$, 1.8 ± 0.35 *vs.* 38.3 ± 5.79µmol/l). The animals treated with nicotine 130µg/kg/h presented a significant decrease in glutamate (68% decrease from end of hypoxia) compared to the saline treated animals (17% increase from end of hypoxia) at d1 (2 hours after hypoxia) ($p = 0.002$), but not for d2 (end of experiment, 4 hours after hypoxia).

From baseline to the end of hypoxia there was a significant rise in NPBI in cortex in all the animals ($p < 0.001$, 0.7 ± 0.04 *vs.* 1.1 ± 0.08µmol/l). There was a decline in

cortical NPBI values at d1 for all the groups except the saline treated group (31% increase from end of hypoxia), significantly so for adrenaline (32% decrease) and nicotine 130µg/kg/h (25% decrease from end of hypoxia) ($p = 0.003$ and $p = 0.013$, respectively). There were no significant differences at d2.

These findings suggests that nicotine can decrease striatal glutamate levels after a hypoxic-ischemic insult, supporting the hypothesis that short term, low dosed, nicotine treatment decreases glutamate levels. The decline in extracellular NPBI in cortex supports the findings from in vitro studies, that nicotine chelates free iron. The one hour infusion of low dose nicotine had significant effects two hours after hypoxia, indicating an effect on the early stages of the secondary phase of injury. One could speculate if a prolonged infusion could give a longer lasting effect. These findings support the hypothesis that nicotine has similar effects in a neonatal model of hypoxic-ischemic brain damage as shown in previous experimental settings, suggesting possible neuroprotective effects of nicotine for this cohort.

Paper IV

Nicotine increases the expression of Brain-Derived Neurotrophic Factor mRNA and protein in the hippocampus of hypoxic newborn piglets.

Real time PCR was used to assess the expression of BDNF, AIF and caspase-3 mRNA in the hippocampus, and immunohistochemistry was used to assess the amount of BDNF protein in the dentate gyrus of the hippocampus.

Newborn piglets treated with nicotine 130µg/kg/h presented significantly more BDNF mRNA ($p = 0.029$) and protein than animals treated with saline (33 ± 14 *vs.* 17 ± 8% positive cells, $p = 0.009$). Regarding AIF and caspase-3 mRNA there were no differences between the groups, and we speculated that this might be due to a too short observation period, and that we thus could not draw any conclusions on

nicotine's effects on apoptosis after this experiment. We concluded that nicotine 130μg/kg/h, infused over one hour after global hypoxia in neonatal piglets, increased levels of both BDNF mRNA and protein in the hippocampus. This might imply neuroprotective effects of nicotine in asphyxiated neonates.

5. General discussion

5.1 The need for intervention.

Despite extensive research on prevention and intervention, perinatal asphyxia remains one of the major contributors to perinatal mortality and morbidity (6, 7). Interventional strategies should aim at ameliorating the secondary brain injury, be easily administered, effective when given after delivery, and cost-effective. So far the most promising intervention is therapeutic hypothermia (56). Hypothermia has however not proven able to fully prevent the secondary damage inflicted, and therefore there is still an ongoing search for further options. As mentioned in the introduction there are several groups investigating different agents, with erythropoietin and xenon gas as examples of two promising strategies. The future solution might very well be a combination of several agents, with synergistic effects, as it has already been shown in rodent models for the combination of hypothermia and xenon gas (65, 224). There is a definite need for interventional strategies for this group of patients, to improve prognosis and lower morbidity.

5.2 The role of oxygen

The most common intervention in newborns is the assistance to start breathing, which is required in 10% of all neonates. The guidelines for resuscitation do not give strict advice concidering what oxygen concentration to use when ventilatory assistance is required, and this is an area of ongoing research and debate. Recent reviews and original articles have added further knowledge to the debate, disputing the use of 100% oxygen, both in resuscitation and in the neonatal intensive care unit (44, 175, 225, 226). It should be kept in mind that oxygen is a drug, and should be administered as such, upon indication, and in a dose-response related manner.

5.3 Nicotine as an interventional strategy

This work has aimed at investigating nicotine as a possible neuroprotective agent in hypoxic newborn piglets. Nicotine is an unconventional candidate for neuroprotection in neonatal medicine, since it is mainly associated with maternal smoking and the subsequent impact on neonatal morbidity and SIDS. Nicotine has however in the last two decades proven to be an anti-inflammatory and anti-apoptotic agent in several studies in animal models, on cell-cultures, and even in neonatal animal models (122, 137). As stated in the introduction, under mechanisms of perinatal asphyxia (chapter 1.1.5), an extensive cascade of events occurs during and after a hypoxic-ischemic event. Nicotine has potential effects on several of these, indicating possible potential in limiting and preventing the damage inflicted.

Nicotine has impact on the free radical production by acting both directly on the mitochondria (121, 148), which are crucial in this process, and on the Fenton reaction (154); decreasing the production of free radicals. Another crucial event in the cascade is the calcium overload (16). Nicotine has effect on the levels of calcium, and has been found to reduce intracellular calcium concentrations (138), indicating a possible effect in this phase. The glutamate release is a major event in the sustaining of influx of electrolytes and water into the cells, causing cytotoxic edema and subsequently cell death. Nicotine has been found to lower levels of glutamate (119), and might have a positive effect because of this. The degree of apoptosis could be lowered due to nicotine's anti-apoptotic effects (138, 150), and this way nicotine could prevent some of the secondary injury inflicted after perinatal asphyxia. Regarding the inflammation seen in perinatal asphyxia nicotine could have positive effects. Much research has been done on the so-called 'nicotinic anti-inflammatory pathway', and nicotine has been found to have anti-inflammatory effects both peripherally and centrally (160, 227). Finally there is the effect of nicotine on neurotrophic factors (118, 159). This could be of major impact, especially since BDNF, which has proven to be an effective agent when administered locally (87), can not cross the BBB, and thus is not suitable for

treatment in neonates. Nicotine leads to an increase in BDNF, and this could contribute to neuroprotective effects of nicotine in this group of patients.

5.3.1 Age-related differences in nicotine effect

Concern has been raised regarding difference in mechanisms of effect in adult and neonatal models, with Laudenbach et al (137) finding that stimulation of the nAChR α7 subunit in neonatal mice is detrimental, whereas several of the studies in adult models find this receptor to be the main source of effect (160, 228). Laudenbach et al do, however, find beneficial effects of stimulation of the α4β2 subunit. This is the same subunit that is thought to be the most common within the mammalian brain, greatly contributing to neuroprotection in striatal and cortical neurons (147, 159, 229). To our knowledge there has only been one study conducted on specific receptors in neonatal models, and this should clearly be investigated further.

5.3.2 Dose-related differences in nicotine effect

Nicotine's effects are dose-dependent (227, 230). There are two main theories on why the higher doses show opposite effects to the lower ones. One is the possible desensitization of the nAChR's (229). The other is that high doses of nicotine may allow too much calcium into the cell through the nAChR's and exacerbate cell death (132). Studies published on nicotine present a large variety of doses and dosing regiments. In general one could say that they show opposite effects of high-dose *vs*. low-dose and acute/subchronic *vs*. chronic nicotine administration. There are however differences in the perception of what a 'low dose' is, and what should be classified as acute and chronic treatment. We here summarize some of the findings and the differences in dosing.

Barros et al (230) gave adult rats nicotine 0.3 or 1.0 mg/kg twice daily for nine days and found increased oxidative stress and DNA damage in the hippocampus of the group treated with the higher dose. Bhagwat et al (231) looked at a similar dose

(1.6mg/kg/d for 10 days), finding increased oxidative stress in adult rat brain, liver, and lung. Ryan et al (229) treated adult mice for 14 days with low doses (defined as 0.75 and 1.5mg/kg/d), or high doses of nicotine (defined as 3.0 and 30.0 mg/kg/d); or with a single dose of 1.0mg/kg. They presented protective effects (preventing neurodegeneration) of the acute treatment and of chronic treatment in low doses. The higher doses failed to show any neuroprotection. The doses used in studies showing neuroprotective effects range from 0.35mg/kg as a single dose in adult rats (125); 0.1 mg/kg/h for 12hours x 3 in Laudenbach et al's study in neonatal mice (137); 2.3 mg/kg/d (0.2μmol/kg/h) for four weeks administered to the mother of suckling asphyxiated neonatal rats (122); to the rather high dose of 7mg/kg as a single dose in adult rats in the same study where they found the effects of the low dose (0.35mg/kg) (125). This illustrates the wide range of dosing, and dosing regiments, highlighting the difficulty in finding the optimal dose for nicotine as a neuroprotective agent. Our results indicate that a dose of 0.26mg/kg/h for one hour might be too high to have neuroprotective effects in a neonatal piglet model of hypoxic-ischemic brain damage. We also present data suggesting that it might be of advantage to give the nicotine infusion over a longer time-period than one hour.

5.3.3 What is new in our research on nicotine

To our knowledge this is the first work done on neuroprotective effects of nicotine in neonatal piglets, and shows that our model can be useful in this kind of research. Despite differences between adult and neonatal receptor response to nicotine administration, as shown by Laudenbach et al (137), we have found indications of neuroprotective effects in our model. We have used a lower dose and a shorter dosing regime than the comparable studies, and have thereby contributed to the discussion regarding what dose to use. We have found better effects of 0.13 vs. 0.26 mg/kg/h, and we have also shown that it is unlikely that the systemic activation of the sympathetic nervous system contributes to the positive effects.

5.4 Considerations

Animal models are of great importance in medical research, but have the drawback that they are not directly translational to human situations. Findings in other species can never be directly transferred to humans, and models will always be simplifications of very complex situations.

Our animals received anesthetics that could aggravate the situation, but also in some cases improve it (i.e. barbiturates and their neuroprotective abilities (200)).

Properly assessing neuroprotective treatments requires time. Survival studies would have given valuable information of the actual effects over a longer time span. With the current model that was not possible, and we can therefore only speculate regarding the long-term effects of treatment. We do however consider our model to be a good model for studying the current hypothesis, due to the advantages of comparable anatomy, brain-maturation, physiology and response to hypoxia between newborn pigs and humans (178-180).

Drawbacks of the current model are for one the fact that our animals were to some extent already adapted to extra-uterine life when exposed to hypoxia; and also the fact that the initiation of intervention in papers III and IV was five minutes after the end of hypoxia, which would be difficult in a clinical situation.

Although data from animal models of asphyxia do not necessarily fully mimic the human perinatal conditions, they can hopefully provide us with important understanding of mechanisms of injury and interventions following asphyxia. And we thus hope that our findings, despite shortcomings, can contribute to the understanding and further research on asphyxia, and the possible advantages of nicotine infusion.

5.5 Implications for further research

This study raises several questions regarding the potential use of nicotine in the neonate exposed to oxygen deprivation. Future research should aim at mapping more of the mechanisms behind its neuroprotective effects in the neonatal brain, with a special interest in the different subunits of nAChR's and the subsequent effects of their activation. An important issue is the observation time. To properly assess intervention in perinatal asphyxia, survival studies are required.

Our study has given an indicator of doses, but dose-response studies are needed. Further research should be done with lower doses than ours, since the levels of blood nicotine concentrations differ extensively between our study and that of Chen et al (122) (65 ± 5 *vs*. 5.4 ± 0.7 ng/ml). They show beneficial effects in asphyxiated neonatal male rats. Our study has also shown that a longer infusion time could be beneficial, since the one hour infusion failed to give a prolonged effect on NPBI and glutamate (paper III).

There is clear evidence that there is a difference in how the genders respond to hypoxia. Renolleau et al (232) show that in male animals apoptosis is predominantly carried out through the caspase-independent pathway (AIF), whereas in female animals apoptosis is carried out through the activation of caspase 3. This implies that all neuroprotective strategies should be studied for the two sexes separately.

5.6 From animal studies to clinical use

In adults nicotine has been, and is currently being, tested in clinical settings. It is showing promising effects in Parkinsons disease (233) and ulcerative colitis (165). For ADHD a resent clinical trial found improvement in cognitive performance following nicotine administration in young adults (234). Wittebole et al found

positive effects on the response to endotoxin in young adults who were pretreated with nicotine (167).

Most studies on nicotine concentrate on the nAChR's. If nicotine's effects were carried out solely through effect on these receptors, it would make more sense to use specific nAChR agonists as therapeutic agents rather than nicotine. As stated in the introduction, nicotine does however carry out its effects in more ways than receptor stimulation, and it therefore seems reasonable to focus research on nicotine rather than nAChR agonists, as long as there is no evidence that the effects sought after come from receptor stimulation alone.

Clinical studies of nicotine as a neuroprotective agent in neonates will not be feasible until more research has been done on dose-response, mechanisms, and on long-term effects.

Regarding the use of oxygen in neonatal resuscitation, the findings in this study adds to the evidence against use of 100% oxygen in resuscitation of the neonate. Reminding us that oxygen is a drug, which should be used with consciousness and caution.

6. Conclusions

1. Resuscitation with 21% oxygen inflicted less necrosis than 100% oxygen, supporting other studies in the conclusion that 100% oxygen should not be used routinely for neonatal resuscitation (paper I).

2. Nicotine administered prior to global hypoxia enhanced newborn piglets' ability to endure hypoxia. Despite nicotine's neuroprotective abilities it was not able to counteract the harmful effects of resuscitation with 100% oxygen (paper I).

3. Protective effects of nicotine in doses of 130µg/kg/h and 260µg/kg/h can probably not be explained by a systemic activation of the sympathetic nervous system, since infusions with these doses did not present any increase in plasma catecholamines (paper II).

4. Treatment with nicotine 130µg/kg after global hypoxia induces a significant decrease in striatal glutamate and cortical NPBI two hours after a hypoxic-ischemic insult. Our findings support the hypothesis that nicotine has similar effects in a neonatal model of hypoxic-ischemic brain damage as shown in experimental settings with adult animals. This suggests possible neuroprotective effects of nicotine in neonates (paper III).

5. Treatment with nicotine 130µg/kg/h after global hypoxia increases the expression of BDNF mRNA and protein in the hippocampus. In our model, with an observational period of four hours, it does not have an impact on either the caspase-independent, or –dependent pathways. These results suggest a possible neuroprotective effect of nicotine in the hippocampus of neonates through the up-regulation of the neuroprotective BDNF (paper IV).

In summary, our findings indicate possible neuroprotective effects of nicotine administration in asphyxiated newborns and show that resuscitation with 100% oxygen may cause increased cerebral necrosis.

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